

TEMPERATURE AND WATER AVAILABILITY AFFECT DECREASE OF COLD HARDINESS IN THE APPLE SNAIL, *POMACEA CANALICULATA*

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ABSTRACT

The apple snail *Pomacea canaliculata* is a freshwater snail originating in tropical and temperate South America. We investigated the effect of temperature acclimatization, moisture levels, and food availability on the loss of cold hardiness in overwintering snails. Cold hardiness broke down by four days in snails maintained in aquatic condition at 25°C and by eight days at 20°C. However, snails held at 15°C retained their cold hardiness even after 64 days. In addition, the cold hardiness of snails kept moist (wrapped in wet towel) was maintained for at least 64 days even at 25°C. These results indicate that warm temperature together with increase of water availability decreases the cold hardiness of overwintering snails. Glycerol content reduced as cold hardiness decreased; supporting a previous hypothesis that glycerol has a major physiological role in the development of cold hardiness in this species.

Key words: Ampullariidae, invasive species, deacclimatization, glycerol, glucose.

INTRODUCTION

Cold hardiness, "the ability of an organism to survive at low temperature" (Leather et al., 1993), is a key trait for many ectotherms in temperate and cold region to survive cold seasons (Block, 1982; Lee & Denlinger, 1991; Ansart & Vernon, 2003). Ecological, behavioral and morphological strategies to cold seasons are known in invertebrates (Lencioni, 2004; Block, 1982), and physiological change (e.g., increase of sugars, antifreeze proteins and polyols) to increase their cold hardiness is well studied especially in insects (Lee & Denlinger, 1991). These physiological changes are usually caused by such environmental cues as temperature, day length, moisture, and salinity (Leather et al., 1993; Ansart & Vernon, 2003).

Some molluscs increase their cold hardiness during cold weather (Ansart & Vernon, 2003). Several environmental factors enhance this ability. For example, cold acclimatization seems to be a major factor increasing the cold hardiness of an intertidal snail, *Melampus bidentatus* (Loomis & Hayes, 1987), and a terrestrial snail, *Anguispira alternata* (Riddle & Miller, 1988).

Photoperiod and starvation are known environmental cues that increase the cold hardiness of the land snail *Helix* [*Cornu*] *aspersa* (Biannic & Daguzan, 1993; Ansart et al., 2001, 2002), and the cold hardiness mechanism of this species changes from freezing avoidance to freezing tolerance with increasing body size (Ansart & Vernon, 2004). A change in seawater salinity also increases the cold hardiness of some kinds of intertidal snails (Murphy, 1979). Although factors that enhance cold hardiness before winter are well studied, factors decreasing it are poorly understood.

The apple snail *Pomacea canaliculata* (Gastropoda: Ampullariidae) is a freshwater snail originating in tropical and temperate South America (Martin et al., 2001; Cowie, 2002). It was initially introduced into Asian countries as a source of human food. However, commercial markets failed, and escaped snails invaded rice paddies, becoming an important rice pest in many countries (Halwart, 1995; Wada, 2004; Cowie et al., 2006; Hayes et al., 2008). After the introduction this snail into temperate Japan in 1981, it became a pest of rice and an important constraint to the spread of direct sowing technology (Mochida, 1991; Wada et al., 1999; Wada,

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2004). Overwintered snails feed on sprouts or rice seedlings and seriously restrict seedling establishment, especially after heavy rain.

A recent study revealed that *P. canaliculata* increases its cold hardiness before the onset of winter (Wada & Matsukura, 2007), and cold acclimatization was a major factor enhancing it. Desiccation also enhanced it to an extent, and starvation might have increased it. However, photoperiod did not influence it (Matsukura & Wada, 2007). Glycerol content increased with the development of cold hardiness, and glucose content showed temporal increase (Matsukura et al., 2008). Both molecules are well known to increase cold hardiness in insects (Lee & Denlinger, 1991; Leather et al., 1993).

The purpose of this study was to determine the effects of temperature, water condition, and starvation on decreasing the cold hardiness of overwintering *P. canaliculata*. These ecological factors are known to influence the increase of cold hardiness in this species (Matsukura & Wada, 2007). It is necessary to understand how cold hardiness decreases as well as increases in order to fully understand the ecological and physiological mechanisms of cold hardiness change in *P. canaliculata*. This study is the first to experimentally determine the environmental factors that lower cold hardiness in molluscs. In addition, we examined the changes in glycerol and glucose contents during the loss of cold hardiness.

MATERIALS AND METHODS

Snails

We collected snails found resting on the sediment of channels around the lotus paddy fields in Kumamoto city, Kumamoto Pref., Japan (32.5°N, 130.4°E), on 1 December 2006. We used only juveniles with shell heights from 7.5 to 17.5 mm, which constitute the majority of overwintering snails in paddy fields (Wada et al., 2004). The cold hardiness of *P. canaliculata* is fully developed by December (Wada & Matsukura, 2007). Thus, the snails collected were cold-tolerant ones; we confirmed this with a mortality test.

Cold Treatment to Evaluate the Cold Hardiness of Snails (Mortality Test)

We evaluated the cold hardiness of the snails by the method as described in Wada & Matsukura (2007). Twenty snails (basically, five replicates)

were wrapped in a moist towel, confined in a plastic cup (10 cm diameter, 4 cm deep), and held at 0°C for five days. Cold-intolerant snails (collected in summer) cannot survive this treatment, but most cold-tolerant snails (collected in December) can (Wada & Matsukura, 2007). Thus, cold-tolerant populations can be clearly distinguished from intolerant ones. After the treatment the snails were immersed in tap water for 24 h at room temperature (17–22°C). Snails that everted a foot were regarded as survivors.

Environmental Treatment 1: Temperature

Snails were reared at 25, 20, or 15°C. They were confined in a plastic container (18 cm × 30 cm, 20 cm deep; 100 snails per container) filled with ample water (approximately 15 cm deep) and given a cabbage leaf as food. The containers were kept under long-day condition (16 h light, 8 h dark). At 25°C, 100 snails were taken out of the container at 0.25 (6 h), 0.5 (12 h), 1, 2, and 4 days, respectively (totally 500 snails used), then cold-tested (mortality test). At 20°C, snails were taken out and tested at 0.25, 1, 4, and 8 days. At 15°C, snails were sampled as at 20°C and at 64 days. Thus, a total of 1,400 snails were used in this experiment.

Environmental Treatment 2: Water Condition

Because apple snails hibernate in water or in the soil of drained paddy fields, we compared the cold hardiness of snails between two water conditions: in aquatic and moist conditions. Twenty snails were wrapped in a moist towel, confined in a plastic cup, and held at 25°C as above. Survival was checked by mortality test at 1, 4, 8, and 64 days (4 replicates in each sampling date). We compared the survivorship with that at 25°C in treatment 1, which was conducted in aquatic condition.

Environmental Treatment 3: Starvation

A Starvation test in aquatic condition was conducted at 25°C. The 25°C condition in treatment 1 was used as the fed treatment. No food was given in the starved treatment. Survival was checked by mortality test at 0.25, 1, and 4 days (20 snails × 5 replicates in each sampling day).

Chemical Analyses

We measured the glucose and glycerol contents of snail bodies in the 25°C temperature of

treatment 1. Snails were sampled at 0 (control), 0.25, 0.5, 1, 2, or 4 days. The soft body was carefully removed from the shell, weighted (fresh mass: FM), and homogenized in 2 mL 80% ethanol. The tissue was extracted according to Izumi et al. (2005). The glucose was measured by the mutarotase-GOD method (Glucose CII-test, Wako, Japan), as described in Matsukura et al. (2008). The glycerol was measured with an F-kit (Boehringer Mannheim, Mannheim, Germany). Ten snails were used in each measurement.

Statistical Analyses

Analysis of variance (ANOVA) followed by Tukey's Highly Significant Differences (HSD) test at 5% probability level were conducted for multiple comparisons of the survival in the mortality test following the temperature treatment. The *t*-test was used to compare the water conditions and the feeding conditions, and an ANOVA followed by Tukey's HSD test was used to compare glucose and glycerol contents between samples. Pearson's product-moment correlation coefficient (*r*) was estimated to evaluate the interdependence between the survival and the amounts of glucose and glycerol. All percentage data were arcsine square-root transformed before analysis.

Temperatures in Field

We obtained temperature data from Automated Meteorological Data Acquisition System (AMeDAS) point at Kikuchi, Kumamoto Pref. (32.9°N, 130.8°E) operated by the Japanese Meteorological Agency. We calculated the daily average, average of daily maximum, and average of daily minimum temperatures from December to June for 5 years (from 2002–2003 to 2006–2007).

RESULTS

Initial Cold Hardiness of Field-collected Snails

Before any treatments (just after collection), $83.0 \pm 0.04\%$ (mean $\pm$ SE) of snails survived the cold treatment (0°C for five days), indicating that most field-collected snails were cold tolerant.

Changes in Survival in the Mortality Test

Temperatures greatly affected survivals in environmental treatment 1 (Fig. 1). More than 95% of snails held at each temperature

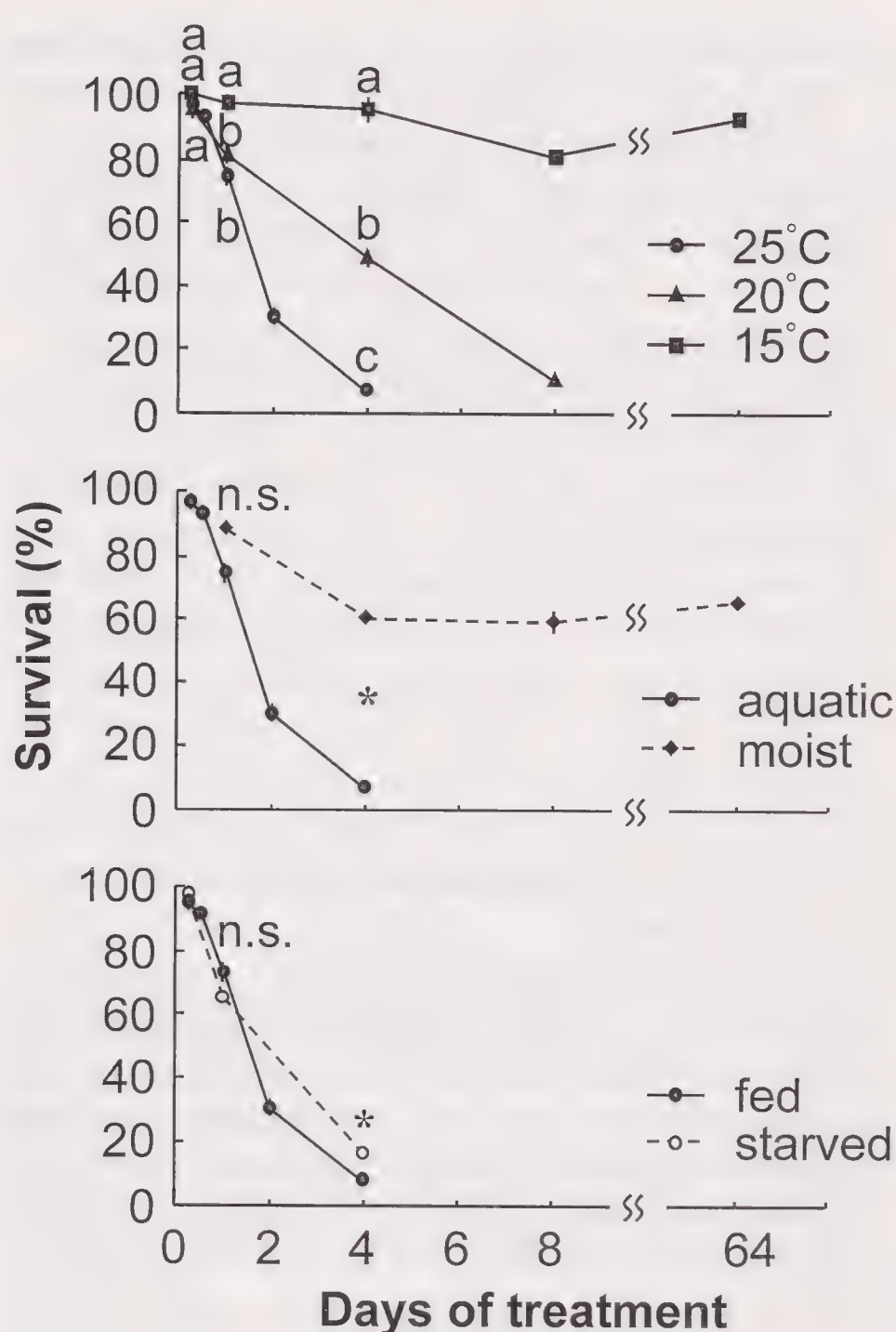


FIG. 1. Changes in survival after cold treatment (0°C for five days) of *P. canaliculata* reared under different environmental treatments: temperature in aquatic condition (top); water condition (center); starvation (bottom). Vertical bars indicate SE; a–c: Different letters indicate significant difference from other treatments on the same sampling date (Tukey's HSD test, $P < 0.05$). *Significant difference between two treatments on the same sampling date (*t*-test, $P < 0.05$).

for 0.25 day (6 h) survived cold treatment, and the difference in survivals depending on temperatures was not clear (ANOVA, $F = 1.229$, d.f. = 14, $P = 0.327$). At one day, however, survivals were affected by water temperatures ($F = 9.542$, d.f. = 14, $P = 0.003$), and survival at 25°C ($74.0 \pm 5.8\%$) and at 20°C ($81.0 \pm 4.3\%$) were significantly lower than that at 15°C ($97.0 \pm 1.2\%$) (Tukey's HSD test). At four days, the difference became more conspicuous ($F = 79.477$, d.f. = 14, $P < 0.001$). Survival was much lower at 25°C ($7.0 \pm 2.6\%$) and 20°C ($49.0 \pm 5.3\%$) than at 15°C ($95.0 \pm 2.2\%$), and the difference in survival was significant between any pair of temperatures (Tukey's HSD test). At eight days, survival at 20°C decreased to $10.0 \pm 2.7\%$. These results indicate almost complete loss of cold tolerance after four days at 25°C

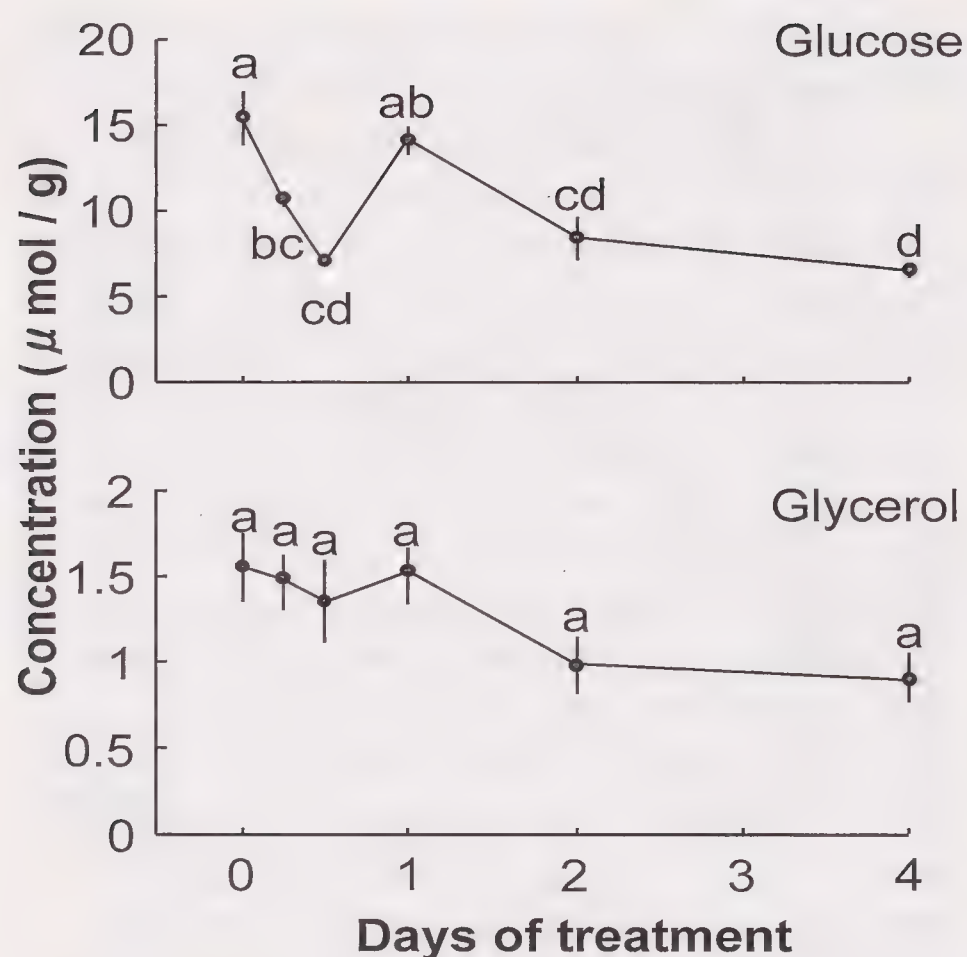


FIG. 2. Glucose (top) and glycerol (bottom) contents during the course of cold hardiness reduction in *P. canaliculata*. Snails were reared at 25°C in aquatic condition with food. Vertical bars indicate SE. Different letters indicate significant difference between samples (Tukey's HSD test, $P < 0.05$). Ten snails were used on each sampling date.

and after eight days at 20°C. On the other hand, snails reared at 15°C retained high survivorship ($92.0 \pm 1.2\%$) until 64 days.

At one day, there was no significant difference in survival of snails after cold treatment between the aquatic ($74.0 \pm 5.8\%$) and moist ($87.5 \pm 3.2\%$) conditions at 25°C (t -test, $t=2.36$, d.f. = 8, $P = 0.137$) (Fig. 1). However, at four days, survival in moist storage ($60.0 \pm 11.4\%$) was significantly higher than that in aquatic condition ($7.1 \pm 2.6\%$) ($t = 2.365$, d.f. = 8, $P = 0.002$). The survival of snails in moist storage remained around 60% up to 64 days, indicating the retention of cold hardiness.

At four days, the survival of starved snails decreased to $16.0 \pm 1.9\%$, and that of fed snails decreased to $7.0 \pm 2.6\%$ (Fig. 1). The survival of starved snails was slightly but significantly higher ($t = 2.31$, d.f. = 9, $P = 0.041$).

Changes in Glucose and Glycerol Contents During Treatment

Glucose contents varied greatly (ANOVA, $F = 15.327$, d.f. = 5, $P < 0.001$), between a maximum of 3.1 ± 0.3 μg/mg FM at 0 days and a minimum of 1.3 ± 0.1 μg/mg FM at four days at 25°C in aquatic condition (Fig. 2). However, the trend lacked consistency, decreasing gradually

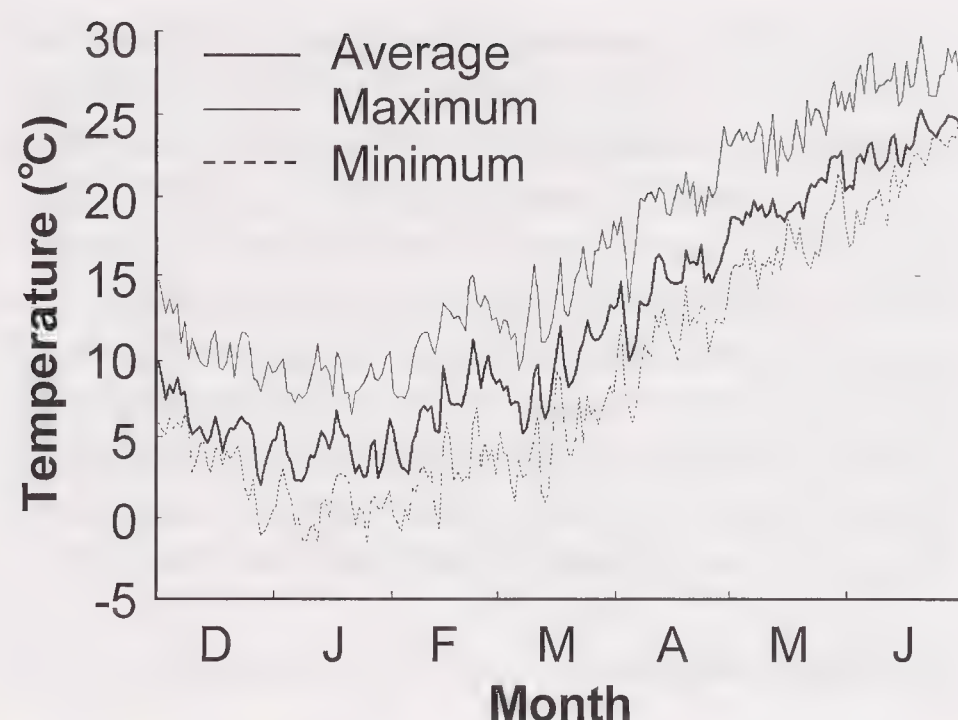


FIG. 3. Daily average, average of daily maximum, and average of daily minimum air temperatures from January to June at Kikuchi, Kumamoto, calculated from the past five years' data (2003–2007).

from 0 to 0.5 days, increasing suddenly at one day, and again decreasing gradually to four days (Fig. 2). There was no correlation between glucose content and survival (Pearson's regression analysis, $r = 0.441$, d.f. = 4, $P = 0.382$).

There was significant change in glycerol content (ANOVA, $F = 2.426$, d.f. = 5, $P = 0.047$), decreasing from a maximum of 0.14 ± 0.02 μg/mg FM at 0 days to a minimum of 0.08 ± 0.01 μg/mg FM at four days (Fig. 2). Furthermore, there was a significant positive correlation between the glycerol content and survival after the mortality test (Pearson's regression analysis, $r = 0.879$, d.f. = 4, $P = 0.021$).

Field Temperature from January to June

The daily average temperature in Kikuchi was under 10°C on most days from December to mid-March, then it increased to 15°C in mid-April (Fig. 3). It reached nearly 20°C in early May and 25°C in mid-June.

DISCUSSION

Warm temperature greatly decreases cold hardiness in *P. canaliculata* in aquatic condition (Fig. 1). Cold hardiness was almost completely lost by four days at 25°C and by eight days at 20°C. Yet it was retained for 64 days at 15°C. These results indicate that the loss of cold hardiness in *P. canaliculata* depends on temperature $\geq 20^\circ\text{C}$ in aquatic condition. Although environmental factors decreasing

cold hardiness in molluscs have not previously been studied, exposition to warm temperature (deacclimatization) is known to lower cold hardiness in some insects (e.g., Rojas et al., 1983; Coulson & Bale, 1990; Ślachta et al., 2002) and plants (reviewed by Kalberber et al., 2006).

Water condition also affected the loss of cold hardiness. Although the snails lost their cold hardiness by four days at 25°C in aquatic condition, approximately 60% survived cold treatment after moist storage for at least 64 days (Fig. 1). These results show that the cold hardiness of *P. canaliculata* is not lost at warm temperature in moist storage. This fact supports the finding that moist storage encouraged cold-intolerant snails to acquire cold hardiness even at warm temperature (Matsukura & Wada, 2007). Cold hardiness and desiccation tolerance of insects often overlap physiologically and ecologically (Ring & Danks, 1994; Danks, 2000). Therefore, the response of *P. canaliculata* to desiccation – in fact, water content of snails decreased during moist condition (Matsukura et al., 2008) – suggests the linkage between cold hardiness and desiccation tolerance.

The starved snails survived the mortality test better than the fed snails at four days, however, difference in survival was slight (Fig. 1). Thus, starvation seems to be a minor factor to affect cold hardiness.

Our results support the previous suggestion that glycerol plays a major role in the cold hardiness of *P. canaliculata* (Matsukura et al., 2008). The glycerol content was positively correlated with survival in the mortality test, although its change was small (Fig. 2). The slight change may be due to the accumulation of glycerol in particular organs of cold-tolerant *P. canaliculata* (Matsukura et al., 2008). On the other hand, glucose content was not correlated with survival, and varied greatly during storage (Fig. 2). This result suggests that glucose does not directly affect the cold hardiness of *P. canaliculata*, but its content fluctuates as a result of the metabolic processes of cold hardiness change, as Matsukura et al. (2008) has suggested.

Our results indicate that the cold hardiness of *P. canaliculata* decreases within a week at 20°C in aquatic condition. This would mean that the cold hardiness of overwintering *P. canaliculata* living in water breaks down from late April to early May, because daily-average temperature reaches approximately 20°C until this period (Fig. 3). However, snails living in drained paddy fields would retain their cold hardiness

until early summer. In Kumamoto, paddy fields are drained in autumn, so snails overwinter in moist or dry condition during winter to spring. In mid- to late June, the fields are irrigated for rice planting, and the cold hardiness of the snails would break down quickly then.

This study and our earlier studies (Wada & Matsukura, 2007; Matsukura & Wada, 2007) clearly revealed seasonality of cold hardiness in *P. canaliculata*. This seasonality may occur because of the relation between cost for acquiring cold hardiness and its benefits. Actually in some insects, synthesis of cold-hardiness-related molecules is reported to be costly, affecting their fecundity (Leather et al., 1993). In *P. canaliculata*, acquiring cold hardiness is probably accompanied by consumption of glycogen reserves (Matsukura et al., 2008). Therefore, *P. canaliculata* may break down its cold hardiness in summer, maximizing multiplication. Physiologically, there may be a trade-off between cold hardiness and heat tolerance. The land slug *Lehmannia valentiana* develops cold hardiness in winter, but its heat tolerance then inversely decreases (Udaka et al., 2008). Therefore, physiological state of cold-tolerant slugs has disadvantage for survivals in summer. Seasonality of cold hardiness in *P. canaliculata* may be attributed to this physiological trade-off.

Recently, new molecular approaches are applied in the study of physiological mechanisms of cold hardiness in insects. For example, replacement of water by glycerol in fat body cells with aquaporin, which is one kind of membrane transporter, prevents freezing injury of cells in *Chilo suppressalis* (Izumi et al., 2006). Unsaturated fatty acids produced by delta 9-acyl-CoA desaturases on fatty acid cells enhance cold hardiness in *Delia antique* (Kayukawa et al., 2007). On the contrary, such approaches have not been adopted to molluscs probably because of difficulty in mass rearing and poorly accumulated information in molluscs. *Pomacea canaliculata* seems to be a suitable animal for investigating the mechanisms, because basic knowledge about the cold hardiness of this species has been gradually made clear (e.g. Wada & Matsukura, 2007; Matsukura et al., 2008; this study). Large numbers of snails can be easily collected from paddy fields, and mass rearing is relatively easier than other molluscs. Further studies on the physiology of the cold hardiness in *P. canaliculata* will help to clarify the inclusive mechanism of cold hardiness in molluscs.

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